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# The structure of *Artemia* sp. haemoglobin

## Cleavage of the native molecules into functional units by limited subtilisin digestion

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Limited subtilisin digestion of the high-$M_r$ haemoglobin of the crustacean *Artemia* sp. results in a series of fragments that are multiples of $M_r$ 16000. Properties such as amino acid composition, iron content, absorption and c.d. spectra of the 16000-$M_r$ functional units strongly resemble those of the intact haemoglobin molecules. The 16000-$M_r$ functional units can bind $O_2$ in a non-co-operative way. They thus represent the structural units from which the globin chains are built up.

The haemoglobins have an extremely wide distribution throughout the animal kingdom. In contrast with vertebrate haemoglobins, those of the invertebrates, which may be intra- or extracellular, show a wide variety in size and molecular architecture (Chung & Ellerton, 1979; Wood, 1980). With the exception of the haemoglobins of the chironomid larvae, practically all other extracellular haemoglobins have a high $M_r$ ($10^5$–$3 \times 10^6$). This is the consequence of aggregation of low-$M_r$ (16000) polypeptide chains (Annelida) or of the presence of high-$M_r$ (>100000) polypeptide chains containing multiple functional units (Mollusca, Arthropoda).

The low-$M_r$ polypeptides with 'haemoglobin' function found in annelids (*Lumbricus*/*Glycera*) and in *Chironomus*, together with the polypeptides with 'myoglobin' function found in the tissue of several invertebrates (*Annadara*, *Busycon*, *Aplysia*), all show a high degree of homology in primary structure with the vertebrate myoglobin–globin family, suggesting a common origin (Goodman *et al.*, 1974, 1975; Fasman, 1976; Dayhoff, 1978; Coates & Riggs, 1981). The high-$M_r$ polypeptide chains found in some mollusc and arthropod haemoglobins are, like the haemocyanins, built up as polymers of structural and functional units. The number of functional units found in these globin chains varies considerably. *Planorbis corneum*, *Helisoma trivolvis* and *Cardida borealis* contain respectively 10, 10–12 and 16 functional units, whereas the globin chains of the arthropods *Lepidurus* and *Daphnia* contain only two (Terwilliger *et al.*, 1977; Terwilliger & Terwilliger, 1978; Ilan & Daniel, 1979; Wood & Gullick, 1979; Dangott & Terwilliger, 1980). These functional units, which can be liberated from the intact molecule by limited proteolysis, represent polypeptide fragments with $M_r$ in the range 15000–17000 containing one haem group. They are able to bind $O_2$ reversibly with a very high affinity but in a non-co-operative way (Chung & Ellerton, 1979; Wolf *et al.*, 1983). They strongly resemble the vertebrate myoglobin type. However, the primary structure and the possible evolutionary relationship of the globin chains with multiple functional units remain to be elucidated.

In the haemolymph of the carapaceless branchiopod *Artemia* sp., three haemoglobin phenotypes were identified (Bowen *et al.*, 1969). All three have a native $M_r$ of 260000 and they exist as heterodimers of two globin chains ($\alpha$ and $\beta$) of similar size ($M_r$ 128000). These globin chains have a minimum $M_r$ of 18000 and a haem-group content of one/17000 $M_r$, suggesting that they contain about eight functional units (Moens & Kondo, 1976, 1977, 1978; Wood *et al.*, 1981; Moens, 1982). In a previous paper the cleavage of the *Artemia* haemoglobins into functional units by limited tryptic digestion was described (Geelen *et al.*, 1982). However, the method used was inadequate to produce sufficient amounts of functional units to allow their further purification. In the present paper the limited subtilisin digestion of the *Artemia* haemo-

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globins is described, confirming that they contain multiple functional units. The method further provided sufficient amounts of them to allow their purification to homogeneity (L. Moens, M. L. Van Hauwaert & G. Wolf, unpublished work).

## Materials and methods

### *Purification of Artemia haemoglobin*

A total haemoglobin preparation was purified from frozen adult *Artemia* sp. (Salines du Midi, Le Grau-du-Roi, France) as described previously (Moens & Kondo, 1978; Geelen *et al.*, 1982).

### *Limited subtilisin digestion*

Limited subtilisin digestion of native haemoglobins was performed in 40mM-glycine/NaOH buffer, pH 10.5, containing 10mM-EDTA (Dangott & Terwilliger, 1980). When samples were to be analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Laemmli, 1970), subtilisin digestion was terminated by acidification with HCl to pH 2 at room temperature, after which the samples were neutralized with Tris base and precipitated with 4 vol. of acetone. Subtilisin digestion on a preparative scale was terminated by the addition of phenylmethanesulphonyl fluoride dissolved in a minimal volume of dimethyl sulphoxide (1 mg/ml of digestion mixture).

### *Separation of fragments*

Haemoglobin fragments were separated by gel filtration on a Sephacryl S-200 column (3.5cm × 100cm) equilibrated in 40mM-glycine/NaOH buffer, pH 10.5, containing 2.6mM-phenylmethane-

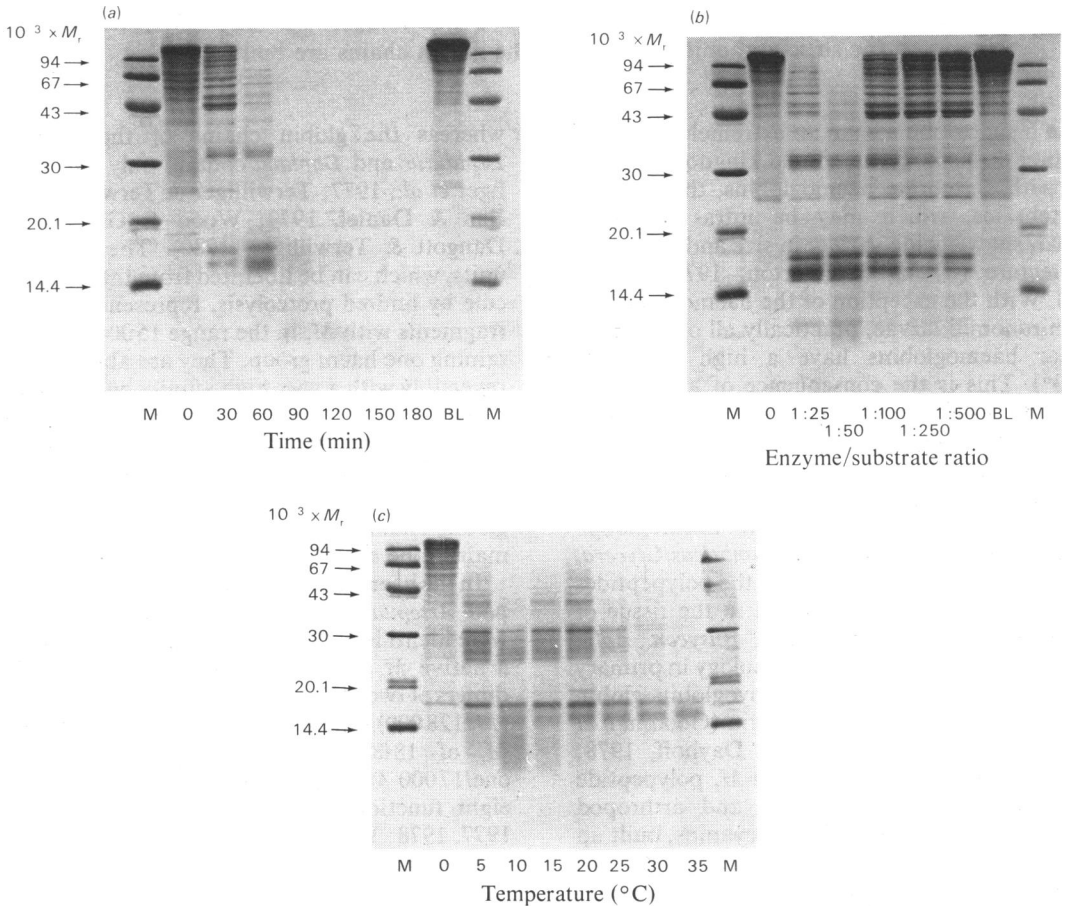


Fig. 1. *Electrophoretic analysis on sodium dodecyl sulphate/15%-polyacrylamide gels by the procedure of Laemmli (1970) of the fragment mixture resulting from limited subtilisin digestion of Artemia haemoglobins*

(*a*) Incubation as a function of time (0–180min) at an enzyme/substrate ratio of 1:50 at 25°C; (*b*) incubation as a function of enzyme/substrate ratio (1:25–1:500) at 25°C for 60min; (*c*) incubation as a function of temperature at an enzyme/substrate ratio of 1:50 for 60min. M, Markers; BL, blank (incubated without enzyme).

Table 1. *$M_r$ values of the fragments resulting from limited proteolysis of Artemia haemoglobins*

| Group | $10^{-3} \times M_r$ as determined by sodium dodecyl sulphate/polyacrylamide gel electrophoresis | Relative concentrations of fragments | | Theoretical size | |
|---|---|---|---|---|---|
| | | Tryptic digestion (Geelen *et al.*, 1982) | Subtilisin digestion (present paper) | Multiples 16000 $M_r$ | $10^{-3} \times M_r$ |
| 1 | 129 | ++ | Trace amounts | 8 | 128 |
| 2 | 112 | + | Trace amounts | 7 | 112 |
| 3 | 97 | + | Trace amounts | 6 | 96 |
| 4 | 84 | +++ | | 5 | 80 |
| | 75 | + | + | | |
| | 72 | + | | | |
| 5 | 67 | +++ | | 4 | 64 |
| | 62 | | + | | |
| | 60 | +++ | | | |
| 6 | 54–55 | ++++ | + | 3 | 48 |
| | 49 | | + | | |
| | 45–46 | + | ++ | | |
| | 41–42 | | + | | |
| 7 | 38 | + | ++ | 2 | 32 |
| | 35–36 | | + | | |
| | 32 | | +++ | | |
| | 29 | | + | | |
| 8 | 26 | | + | – | – |
| | 25 | ++++ | ++ | | |
| | 22 | ++++ | | | |
| 9 | 19 | ++ | | 1 | 16 |
| | 18 | ++ | | | |
| | 17 | ++ | ++++ | | |
| | 16 | ++ | ++ | | |
| | 15 | | ++ | | |
| 10 | ⩾10 several | + | Trace amounts | – | – |
| 11 | Front | ++ | Trace amounts | – | – |

sulphonyl fluoride at a flow rate of 66ml/h. Thyroglobulin, bovine serum albumin, ovalbumin, soyabean trypsin inhibitor and myoglobin were used as $M_r$ markers.

Pooled fractions were concentrated by ultrafiltration.

*Amino acid analysis*

Protein samples were hydrolysed at 110°C in evacuated tubes for 24, 48 and 72h in 6M-HCl. Amino acid analysis was performed with a Jeol 6AH amino acid analyser by the procedure of Spackman *et al.* (1958).

*C.d.*

C.d. spectra were recorded at 20.0°C with a Cary 61 spectropolarimeter (Varian). Measurements in 0.1M-phosphate buffer, pH6.8, were performed at a protein concentration of 1.0–1.5mg/ml in a 5mm cell from 250 to 600nm and with 1:10-diluted samples in a 1mm cell from 200 to 250nm. The full range was 0.02° ellipticity. The ellipticities were converted into molar values $[\theta]$ (250–600nm) or to a mean residue weight (200–250nm). The α-helix content $[f_H]$ was calculated from c.d. data by using the equation $[\theta]_{222} = -30000 f_H - 2340$ (Harrington *et al.*, 1973).

*Iron and protein determinations*

Iron was analysed by electrothermal atomic absorption spectrometry with a Perkin–Elmer 703 instrument equipped with an HGA-500 graphite furnace and deuterium background correction. The method of standard additions (Timm *et al.*, 1980) was used for all the determinations.

Protein concentration was measured by the Lowry method, with bovine albumin as standard.

*$O_2$ binding*

$O_2$ dissociation curves of the haemoglobin fragments were measured in 50mM-Tris/HCl buffer, pH7.5, at 18°C with the diffusion-chamber method (Sick & Gersonde, 1969). Samples (7μl) were loaded into the diffusion chamber and corrections made for the non-negligible thickness of the sample layers (Van Pachtenbeke *et al.*, 1982).

*Other methods*

The other techniques used were as described elsewhere (Moens & Kondo, 1976, 1978; Geelen *et al.*, 1982).

## Results and discussion

*Limited subtilisin digestion of Artemia haemoglobins*

*Artemia* haemoglobin was digested with subtilisin under different conditions of time, enzyme/substrate ratio and temperature. At an enzyme/substrate ratio of 1:50 and at 25°C the haemoglobin substrate was rapidly cleaved into a population of fragments with decreasing $M_r$. Bands with $M_r$ 15000–17000, 30000–32000, 45000–46000 and 64000–68000 are clearly predominant (Fig. 1*a* and Table 1). Although there was only a partial accumulation as a function of increase in time, enzyme/substrate ratio or temperature (Fig. 1), the fragments with $M_r$ 15000–17000 seemed to be relatively the most resistant to subtilisin digestion. The other fragments were more susceptible to proteolysis, their $M_r$ values forming a series of multiples of 15000–17000 (Table 1). The cleavage pattern described is compatible with the hypothesis that the *Artemia* globins, like other invertebrate high-$M_r$ globin chains, are built up of a series of functional units, each forming a compact conformation, linked together by regions of less-folded polypeptide chain that are more susceptible to proteinase degradation than are the functional units themselves (Wood, 1980).

Within the group of 15000–17000-$M_r$ fragments the 15000-$M_r$ polypeptides represent the true structural units from which the *Artemia* globin chains are built up. Fig. 1 clearly shows that their relative concentration increases with increasing proteolytic activity, whereas that of the 17000-$M_r$ fragments decreases. This suggests that the 15000-$M_r$ fragments are formed from the 17000-$M_r$ fragments, which can be considered as the compact functional units and the functional units with linking regions respectively.

The absence of a consistent accumulation of 15000–17000-$M_r$ fragments as a function of incubation time must only be due to a limited resistance of these fragments to proteolysis, creating a temporary steady state. This is illustrated by the progressive decrease in total stainable protein and the overall decrease in $M_r$ of the fragments produced, whereas the relative concentrations of the 15000–17000-$M_r$ fragments are fairly constant (Fig. 1*a*). At higher proteolytic activity, fragments ($M_r$ 12700–13500) smaller than the functional units themselves were frequently observed (Figs. 1*b* and 1*c*). This is most probably the result of cleavage at preferential points within the functional units. Subtilisin digestion of myoglobin under the same conditions produced similar polypeptides ($M_r$ 12500–12800), whereas cytochrome *c* was completely degraded. This suggests a conformation-dependent cleavage and thus a possible similarity in conformation between the vertebrate myoglobins and the functional units of the *Artemia* haemoglobin.

*Separation of the haemoglobin fragments*

The fragment mixtures, obtained after limited subtilisin digestion, were separated on a Sephacryl S-200 column (Fig. 2). Haem-containing fractions with apparent $M_r$ values of 17000–19000 (E), 32000 (D), 50000 (C) and 68000 (B), corresponding respectively to fragments containing a single, two, three or four units, were clearly resolved from undegraded haemoglobin and high-$M_r$ (>68000) fragments (Fig. 2*b*). The relative proportions of the fractions A–E are, as expected, completely dependent on the proteolytic strength used, showing an increase of the lower-$M_r$ fragments with increasing proteolytic activity, confirming the ab-

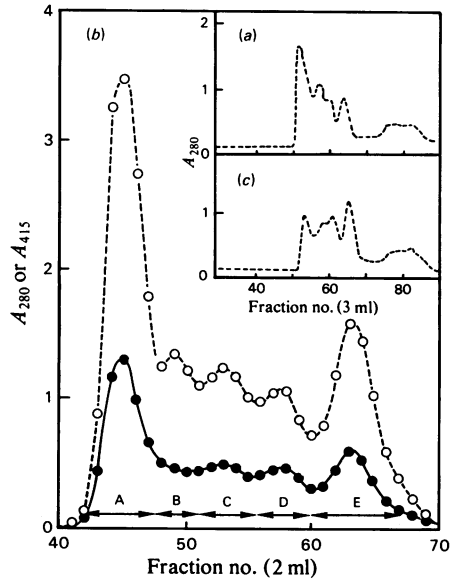


Fig. 2. *Separation by gel filtration of the fragment mixture resulting from limited subtilisin digestion of intact Artemia haemoglobins*

Digestion was performed at an enzyme/substrate ratio of 1:50 for 60min at 15°C (*a*), 25°C (*b*) or 30°C (*c*). The fragments were separated by chromatography on a Sephacryl S-200 column (3.5cm × 100cm) equilibrated in 40mM-glycine/NaOH buffer, pH10.5, containing 2.6mM-phenylmethanesulphonyl fluoride at a flow rate of 66ml/h, and the fractions were pooled as indicated. ○, $A_{280}$; ●, $A_{415}$.

sence of preferential cleavage points (Figs. 2*a*–2*c*) (Geelen *et al.*, 1982). Fractions A–D were subjected to a second subtilisin digestion and rechromatographed under the same conditions (Fig. 3). They produced a set of fragments (A′–E′) that are again multiples of $M_r$ 15000–17000, clearly proving that the *Artemia* globin chains are built up as covalently linked polymers of structural entities of this size.

*Characterization of the haemoglobin fragments*

*Homogeneity.* Fractions A–E are inhomogeneous and represent, as shown by polyacrylamide-gel electrophoresis under denaturing and non-denaturing conditions, collections of polypeptides with similar $M_r$ but differences in charge. This heterogeneity might have been artificially generated by the subtilisin digestion, or may reflect a functional unit and/or linker heterogeneity within the authentic haemoglobin molecules.

As the proteinase cleaves rather non-specifically in the linker regions at one or both ends of the considered fragment, the resulting polypeptides will show similar $M_r$ but possibly charge differences. The functional units and/or the linker regions within a globin chain may or may not be identical in sequence and conformation. Tryptic cleavage clearly showed the existence of a preferential

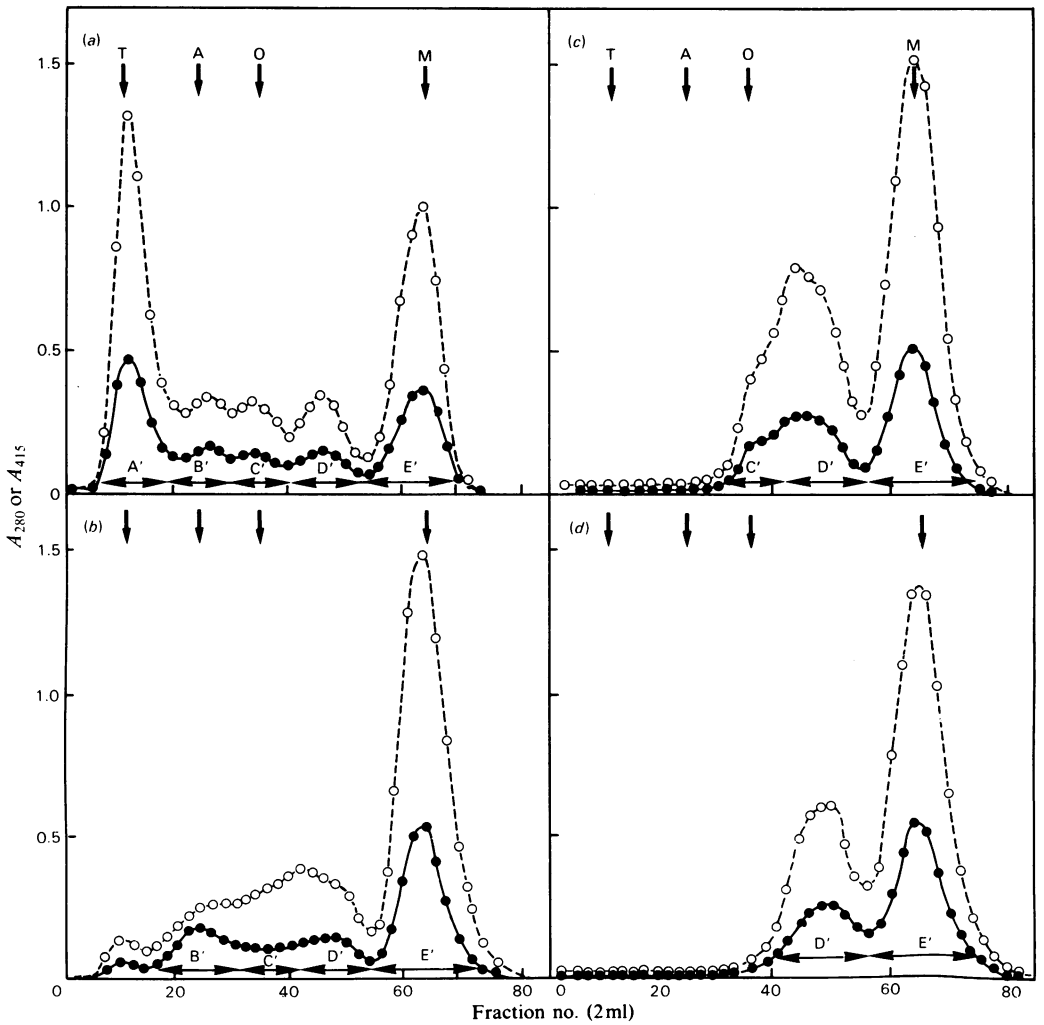


Fig. 3. *Separation by gel filtration of the fragment mixtures resulting from limited subtilisin digestion at 25°C of fractions A–D presented in Fig. 2*

Conditions of digestion and chromatography were as described for Fig. 2. (*a*)–(*d*) show the results for the digests of fractions A–D respectively. ○, $A_{280}$; ●, $A_{415}$. T, A, O and M are the positions of thyroglobulin, bovine albumin, ovalbumin and myoglobin respectively.

cleavage point, confirming the latter supposition. Functional unit and/or linker heterogeneity was suggested for *Planorbis corneus* haemoglobin (Wood & Gullick, 1979) and has been shown to exist in the haemocyanins (Gielens *et al.*, 1977, 1980; Wood, 1980).

*Amino acid composition and iron content.* From the amino acid composition of intact haemoglobin II a minimal $M_r$ of 18000 can be calculated (Moens & Kondo, 1978). The amino acid compositions of fractions A–E are very similar to each other, as well as to those of intact total haemoglobin and haemoglobin II (Table 2), indicating that each fraction contains fragments that are multiples of a structural unit of $M_r$ approx. 17000.

The iron content of the haemoglobin fragments, determined by atomic absorption spectrometry, decreases linearly with decreasing $M_r$. This constant iron/protein ratio (1 Fe atom/16000 $M_r$) suggests that the proteolytic cleavage mainly occurred between the units, leaving the haem environment intact. However, for the 15000-$M_r$ and 32000-$M_r$ fragments a somewhat lower iron content (0.6 Fe atom/16000 $M_r$) than the theoretical value was observed. This indicates that a certain percentage of the fragments had lost their haem group(s), probably by cleavage within the functional units.

*Immunological reactivity.* The immunological reactivity of the different fragments was tested by double immunodiffusion against a purified anti-(haemoglobin II) immunoglobin G (Geelen *et al.*, 1982). The fractions A–E including the single-unit fraction, gave a positive reaction, indicating that they must at least share common antigenetic determinants.

*Absorption and c.d. spectra of fraction E.* The absorption spectra of fraction E in the oxy, deoxy, carbonmonoxy and cyanmet forms are practically identical with those of the intact pigment and human haemoglobin. This strongly suggests that the $O_2$-binding sites of the units are still able to react with different ligands and thus that they are functionally intact despite the subtilisin treatment.

The c.d. spectra in the visible region of the intact molecules and fraction E (oxy form) show, together with some weaker bands, a strong positive and negative Cotton effect in the 400–440nm region, corresponding to the Soret absorption band (Fig. 4*a*). Similar spectra were observed for other invertebrate haemoglobins, whereas vertebrate haemoglobins and myoglobins show only a positive band here (Harrington *et al.*, 1973; Ascoli *et al.*, 1976; Bannister *et al.*, 1976; Wood & Gullick, 1979). The overall shape of the c.d. spectrum in this region is strongly dependent on any modifica-

Table 2. *Amino acid compositions of fragments of Artemia haemoglobin*

Amino acid analysis of total haemoglobin and of the fractions A–E was performed as described in the Materials and methods section. The data are the averages of the values obtained after 24, 48 and 72 h hydrolysis, except for threonine and serine, which were extrapolated to zero time, and for valine, isoleucine and histidine, for which the 72 h-hydrolysis values were used. Abbreviation: N.D., not determined.

| | Composition (%, w/w) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Haemoglobin | | Fraction | | | | |
| Amino acid | II* | Total | A | B | C | D | E |
| Lys | 7.16 | 8.31 | 6.88 | 7.06 | 6.45 | 6.47 | 6.77 |
| His | 4.56 | 3.22 | 3.48 | 3.26 | 3.26 | 2.77 | 3.13 |
| Arg | 8.00 | 7.40 | 8.10 | 7.75 | 7.28 | 7.62 | 7.26 |
| Asp | 9.76 | 9.16 | 10.34 | 10.66 | 11.05 | 11.34 | 10.74 |
| Thr | 4.07 | 4.82 | 4.57 | 4.57 | 3.97 | 4.43 | 4.81 |
| Ser | 4.13 | 4.78 | 4.57 | 4.77 | 4.30 | 4.77 | 5.12 |
| Glu | 12.73 | 12.91 | 13.88 | 13.54 | 12.62 | 14.00 | 12.71 |
| Pro | 3.03 | 3.51 | 3.09 | 3.55 | 3.73 | 3.23 | 3.45 |
| Gly | 3.81 | 4.23 | 4.25 | 4.12 | 4.15 | 4.01 | 4.75 |
| Ala | 5.09 | 5.74 | 5.77 | 5.41 | 5.63 | 6.01 | 5.64 |
| Cys | 0.69 | Trace | Trace | Trace | Trace | Trace | Trace |
| Val | 6.94 | 6.92 | 5.48 | 5.61 | 6.62 | 5.48 | 6.89 |
| Met | 2.04 | 0.69 | 0.79 | 1.80 | 1.04 | 1.21 | 0.66 |
| Ile | 5.17 | 5.72 | 4.43 | 4.43 | 5.36 | 4.75 | 5.23 |
| Leu | 10.63 | 11.22 | 12.96 | 12.28 | 12.79 | 13.34 | 11.24 |
| Tyr | 3.02 | 3.61 | 3.01 | 3.27 | 2.97 | 3.10 | 3.31 |
| Phe | 7.87 | 7.75 | 8.36 | 7.90 | 8.80 | 7.46 | 8.29 |
| Trp | 1.29 | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |

* Amino acid composition of haemoglobin II, from Moens & Kondo (1978).

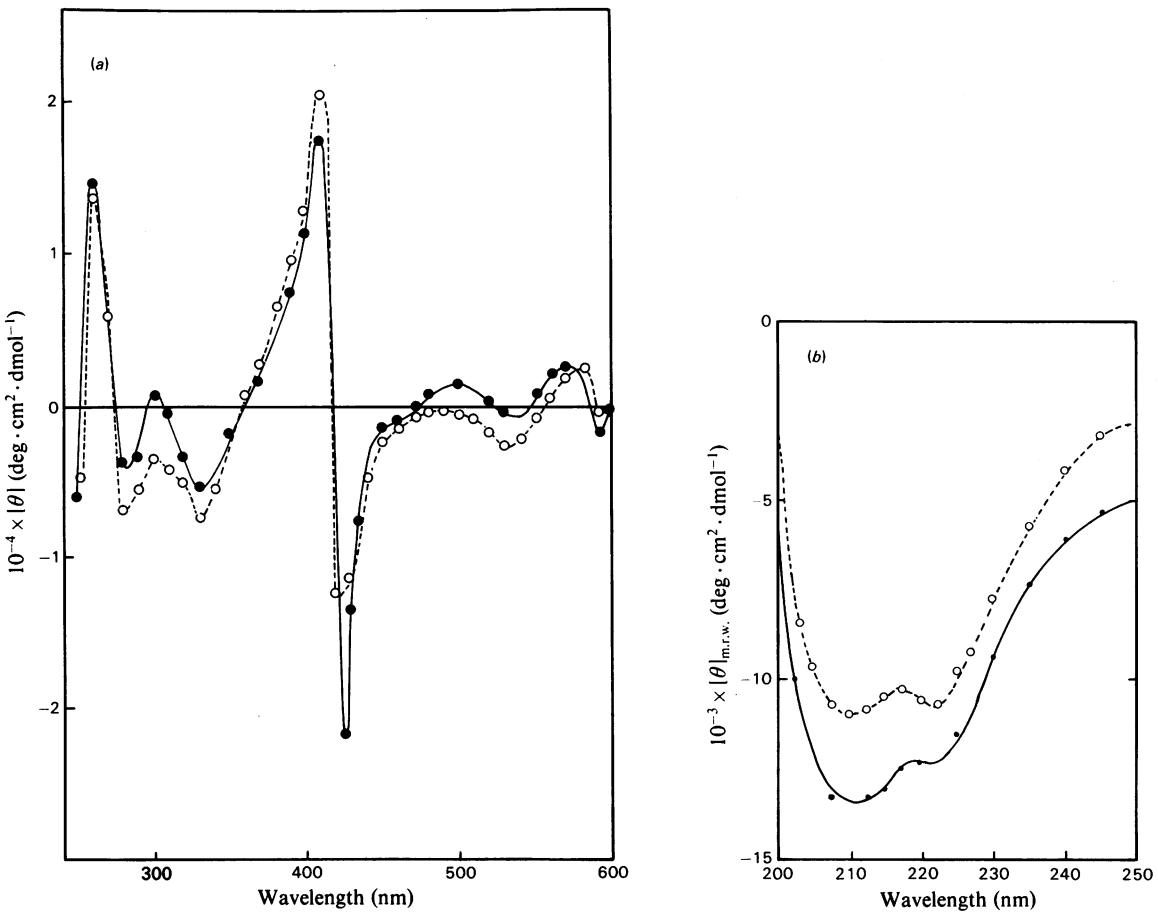


Fig. 4. *C.d. spectra of the oxy forms of intact Artemia haemoglobin and fraction E*
Measurements were performed in 0.1 M-phosphate buffer, pH 6.8, as described in the Materials and methods section. (*a*) Spectra in the near-u.v. and visible regions; (*b*) spectra in the far-u.v. region. ——, Intact haemoglobin. -----, fraction E.

tion of a substituent or of the conformation (Ascoli *et al.*, 1976; Wood, 1980). As structural information is not yet available for these invertebrate globin chains, a precise interpretation of these spectra cannot be given. However, as the overall shapes (position and sign of maxima) of the c.d. spectra of the intact *Artemia* haemoglobin and of fraction E are very similar, the haem environment of the latter must be unaffected by the subtilisin treatment. The observed differences in amplitude at 410 nm and 425 nm are small, and may be caused by the difference in co-operativity between both molecules (Ascoli *et al.*, 1976).

The α-helix contents of the intact molecule and of fraction E (Fig. 4*b*) are rather low (25–35%) compared with those of most other invertebrate haemoglobins (35–75%) (Huber *et al.*, 1968; Swaney & Klotz, 1971; Bannister *et al.*, 1976; Wood *et al.*, 1976). Only *Lumbricus* and *Eunice* haemoglobins showed comparable values (33–45%). However, the values obtained from c.d. spectra for *Lumbricus* haemoglobin (Harrington *et al.*, 1973; Ascoli *et al.*, 1976) are in contrast with those (60–70%) determined for chain $A_{III}$ from sequence data (Garlick & Riggs, 1982). Wood (1980) suggested that this may be attributed to the presence of certain chains with a very low helical content. *Artemia* haemoglobin preparations always contain some percentage of partially degraded molecules, resulting from proteolytic degradation during isolation (Moens & Kondo, 1978; Krissansen *et al.*, 1981). It is possible that the partially degraded molecules have a much lower helicity, probably as a consequence of the loss of haem (Yip *et al.*, 1972), lowering the total α-helix content to the observed values. A similar argument can be

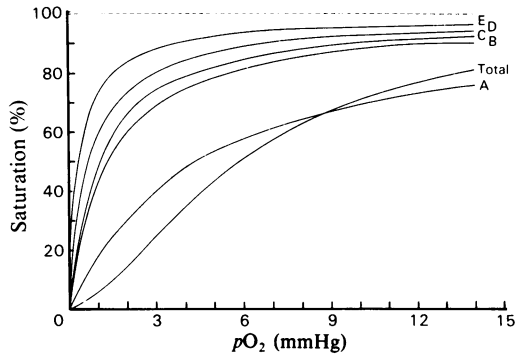


Fig. 5. *$O_2$-dissociation curves of intact Artemia haemoglobin and its fragments*

Measurements were performed in 50mM-Tris/HCl buffer, pH7.5, at 18°C with the use of the diffusion-chamber technique of Sick & Gersonde (1969).

used for fraction E. In the latter case its validity is confirmed by the iron content of fraction E, which is lower than the theoretical value.

*$O_2$ binding*. The $O_2$ dissociation curves of fractions A–E were determined by using the diffusion-chamber technique of Sick & Gersonde (1969) (Fig. 5). The fractions, although proteolytic-degradation products, bound $O_2$ reversibly. Their co-operativity decreased to unity and their $O_2$ affinity ($P_{50}$) increased with decreasing $M_r$. These results are discussed elsewhere (Wolf *et al.*, 1983).

## Conclusion

Although no quantitative data are given on the subtilisin cleavage of *Artemia* haemoglobin, the results presented in this paper are compatible with a random scission of a polymer ($M_r$ 130000) containing eight monomers of $M_r$ 16000 (Tanford, 1961). The $M_r$ of the cleavage products, their Fe/protein ratio and amino acid composition all confirm that the *Artemia* globin chains are built up by the covalent linkage of eight structural units of $M_r$ approx. 16000.

The isolated units (fraction E) are also functionally intact. This is conformed by the comparison of the iron content, absorption and c.d. spectra, and the $O_2$-binding characteristics of the isolated units and the intact molecules (Figs. 4*a*, 4*b* and 5).

The similarity in amino acid composition of total haemoglobin and of fractions A–E clearly shows the absence of specifically localized sequences in the globin chains. This implies that the amino acid sequence of a single unit reflects roughly that of all the units in the globin chains. A comparison of the primary structure of a structural and a functional unit of the *Artemia* giant globins with the vertebrate globin sequences should reveal their structural and evolutionary relationship. However, determination of their primary structures first needs their purification to homogeneity (L. Moens, D. Geelen, M. L. Van Hauwaert & G. Wolf, unpublished work).

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